Journal of Medical Virology 60:256–263 (2000)

Hepatitis B Virus e Antigen Specific Epitopes and Limitations of Commercial Anti-HBe Immunoassays

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Current commercial hepatitis B virus (HBV) anti-HBe immunoassays are designed so that anti-HBe is detectable only in the absence of excess HBeAg. Recently, with the use of direct anti-HBe assays, anti-HBe was detected in individuals who had been seropositive for several years for HBeAg [Maruyama et al. (1993) J. Clin. Invest. 91:2586–2595]. Although anti-HBe seroconversion does not necessarily indicate subsequent HBeAg clearance, the ability to detect earlier anti-HBe seroconversion could have clinical significance for monitoring patients undergoing HBV immunotherapy (e.g., α interferon therapy). Because the HBeAg and the HBcAg share 149 amino acids, an anti-HBe assay must distinguish anti-HBe from anti-HBc antibodies. Although the HBV HBeAg and HBcAg display distinct immunogenic determinants, much remains unknown regarding the complete epitope spectrum specific to each antigen. The goal of this study was 3-fold. The first objective was to identify HBeAg specific linear epitopes. The second objective was to design an anti-HBe immunoassay capable of detecting anti-HBe specific antibody in the presence of excess HBeAg. The third objective was to characterize early anti-HBe seroconversion antibodies. The major linear epitope residing in the HBeAg amino acid sequence was mapped and 2 novel minor epitopes (δ, γ) which appear to be HBeAg specific have been identified. An anti-HBe immunoassay capable of detecting anti-HBe specific antibody in the presence of excess HBeAg was designed. Finally, it was found that early anti-HBe seroconversion antibodies appear to be conformational, whereas later seroconversion, more typically associated with the clearance of HBeAg, is characterized by the presence of antibodies to the linear HBeAg epitopes. ***J. Med. Virol. 60:256–263, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: epitope; seroconversion; reactivity; neutralization

INTRODUCTION

The core gene of the hepatitis B virus (HBV) codes for 2 different proteins, HBeAg, a secreted antigen [Ou et al., 1986], and HBcAg, which makes up the nucleocapsid. HBeAg shares a 149 amino acid sequence with HBcAg, but contains an additional 29 amino acid (aa) N-terminal precore sequence [Standring et al., 1988]. Within the precore sequence is a 19 aa leader sequence, which directs the protein to the endoplasmic reticulum where the leader sequence is removed, leaving a 10 aa precore sequence [Standring et al., 1988]. Before secretion, up to 36 aas of the C-terminus are removed [Standring et al., 1988]. The remaining 10 aas of the precore sequence have been found to be essential for the correct folding of the HBeAg [Nassal and Rieger, 1993]. This sequence contains a cysteine, at position –7, which has been found to form a disulfide bridge with a cysteine at position 61 [Nassal and Rieger, 1993]. The cysteine at position 61 of the HBcAg, however, forms a disulfide bond with a neighboring HBcAg monomer at cysteine position 61 [Nassal and Rieger, 1993]. These HBcAg dimer subunits self assemble to form the nucleocapsid, which is made up of 180 and 240 dimer subunits [Bottcher et al., 1997]. The function of the HBeAg is not understood fully. It has been suggested that the HBeAg protects HBV-infected hepatocytes from immune surveillance via the development of a tolerance to HBeAg and HBcAg [Uy et al., 1986; Milich et al., 1990; Bonino et al., 1991].

Recombinant HBeAg (rHBeAg) has been cloned successfully in both *Escherichia coli* and yeast [Inada et al., 1989; Broker et al., 1993]. Native, serum-derived HBeAg has been found to exist in sera of infected individuals in at least 2 forms, a large molecular weight (MW) form bound to IgG and a small MW, IgG-free polypeptide [Kurai et al., 1991]. It is believed that the large MW form is an IgG immunocomplex consisting of HBeAg and anti-HBe IgG. Serum-derived HBeAg also has been found to exist as a heterogeneous population

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Accepted 21 September 1999

of different lengths ranging from 15 to 18 kDa [Standring et al., 1988].

Because HBeAg and HBcAg share an identical 149 aa sequence, the possibility that the 2 antigens display cross reactivity has long been studied. Early characterization of the HBeAg and the HBcAg indicated that the human antibody response to the HBcAg was primarily conformational, whereas that of the HBeAg was primarily linear [McKay et al., 1981]. A more detailed characterization of HBeAg has revealed the existence of 3 immunogenic determinants recognized by human antibodies: 1 linear region spanning aas 76–89 termed "HBe1" [Salfeld et al., 1989], 1 conformational region spanning aas 2–140 termed "HBe2" [Salfeld et al., 1989], and 1 linear region spanning aas 128–133 termed "β" [Sallberg et al., 1993]. Further characterization of the HBcAg has revealed that the human antibody response to the HBcAg is primarily conformational and directed against a single immunogenic region in the vicinity of aas 74–89 [Ferns and Tedder, 1986; Salfeld et al., 1989]. It also has been found, however, that the human antibody response to the HBcAg is directed against many linear epitopes, including aa region 74–84 [Tordjeman et al., 1993].

According to these findings, human antibodies against the HBcAg and the HBeAg recognize the linear regions 74–84 and 76–89, respectively. This would imply a shared antigenic region between the 2 antigens encompassing the region 76–84. Despite the known immunogenic distinctions between the 2 antigens, a significant gray zone in the human antibody response specific to each antigen remains.

HBeAg appears normally during the stage of high infectivity in the serologic profile and is an indicator of active viral replication. Hence, serologic tests that detect the presence of HBeAg and anti-HBe have a clinical significance. Recently, HBV-infected patients receiving interferon therapy have been monitored for the presence of HBeAg and anti-HBe. A reduction in serum HBeAg levels in conjunction with seroconversion to anti-HBe indicates a positive response to the therapy and a positive prognosis for the patient [Evans et al., 1997].

Two of the most prevalent commercial anti-HBe immunoassays are the Abbott Laboratories (North Chicago, IL) and the Sorin anti-HBe EIAs. Abbott Laboratories employs human anti-HBe for capture and detection of HBeAg. Sorin (Sienna, Italy) uses a monoclonal anti-HBe capture antibody that recognizes the HBe1 epitope and a monoclonal anti-HBe detection antibody that recognizes the HBe2 epitope [Salfeld et al., 1989]. These assays are designed such that anti-HBe is not detectable in the presence of excess HBeAg [Maruyama et al., 1993]. The ability to detect anti-HBe in the presence of excess HBeAg, however, should have a clinical significance, as seroconversion to anti-HBe has a prognostic value.

The goal of this study was 3-fold. The first objective was to characterize the HBeAg in order to identify the anti-HBe specific linear epitopes recognized by human anti-HBe. The second objective was to design an anti-HBe immunoassay capable of detecting anti-HBe specific antibodies in the presence of excess HBeAg. The last objective was to characterize early anti-HBe seroconversion antibodies.

MATERIALS AND METHODS

HBeAg Construct

The rHBeAg construct was cloned and expressed in yeast strain AD2. The plasmid used was ps.PE.P144. This recombinant antigen was constructed using human superoxide dismutase (SOD) fused at the N-terminus. The SOD fusion antigen was used to increase expression [Hallewel et al., 1985, 1989]. The construct contained the 10 aa precore sequence followed by aas 1–144 of the HBeAg sequence subtype *adw*. The rHBeAg was purified as follows. Yeast cells were broken in a Dynomill, centrifuged, and the pellet was retained. The pellet was washed with 1 M urea/lysis buffer, centrifuged, and the pellet and supernatant were retained. The pellet was washed again with 2 M urea/lysis buffer, centrifuged, and the pellet and supernatant were retained. The 1 M and 2 M urea supernatants were combined and a 30% ammonium sulfate cut was performed. This was followed by centrifugation, and the pellet was retained. The pellet was resolubilized in 8 M urea/50 mM Tris, pH 8.0. This was followed by dialysis against 25 mM MES/3 M urea, pH 5.8, overnight at 4°C. After centrifugation the supernatant was retained. This supernatant was diafiltered into a S Sepharose ion exchange column (IEC) equilibration buffer (50 mM MES, pH 5.8, 3 M urea, 10 mM CHAPS, 1 mM EDTA) at 4× on an Amicon (Beverly, MA) YM-10 membrane. The sample was then diluted 1:8 with this IEC equilibration buffer and loaded onto a S Sepharose IEC column at 4°C. The column was washed with this equilibration buffer until an optical density (OD) of 0.00 was achieved. The column was then eluted with a 0–0.5 M NaCl gradient in the IEC equilibration buffer. Fractions were collected and the peak was concentrated on a YM-10 membrane. An S 300 Sephacryl gel filtration column (GFC) was equilibrated with a buffer consisting of 50 mM MES, pH 5.8, 6 M urea, 0.3 M NaCl, 5 mM CHAPS, 5 mM EDTA, and 5 mM DTT. Urea and DTT were added to the peak to 8 M and 10 mM, respectively, and loaded onto the S 300 Sephacryl GFC column at room temperature. Polymerized and monomeric fractions were collected separately. The monomeric form was used for this study.

Epitope Map

The major linear epitopes of the HBeAg were mapped using 147 overlapping octapeptides in a pin solid-phase pepscan study [Chien et al., 1994]. The HBcAg sequence subtype *adw* from aas –10 to 137 was scanned against the Abbott anti-HBe:HRP conjugate contained in the Abbott anti-HBe/HBe EIA kit.

Peptides

The peptide spanning aas 74–89, purported to contain linear epitopes recognized by both human anti-

HBc specific antibodies [Tordjeman et al., 1993] and human anti-HBe antibodies [Sallberg et al., 1993], was synthesized. Peptides containing aa sequences 48–57, 88–99, and 119–134 recognized by human anti-HBe according to our epitope map were also synthesized. All peptides were synthesized according to the HBeAg *adw* subtype amino acid sequence by SynPep Corp. (Dublin, CA).

Human Sera

Various HBV HBsAg positive human samples were purchased from Bioclinical Partners (Franklin, MA) and Boston Biomedica, Inc. (W. Bridgewater, MA). An HBV mixed titer panel used in this study was purchased from Boston Biomedica, Inc. Seroconversion panels were purchased from Bioclinical Partners and Profile Diagnostics, Inc. (Sherman Oaks, CA).

Production of Monoclonal Antibodies (mAbs)

mAbs were produced according to standard procedures. Mice were immunized with rHBeAg (a gift from Darrell Peterson of Virginia Commonwealth University). Four clones (8C71D3, 8A5, 8C72D2, 7D10), produced by Caltech (Pasadena, CA), were selected which produced antibodies specific for rHBeAg. Anti-HBcAg (mAbs) were also produced by Caltech by immunizing mice with particulate rHBcAg (Chiron, Emeryville, CA) and 2 clones (48F, 3A3) were selected. Mice were also immunized with rHBeAg ps.PE.P144 (Chiron), and 4 clones (2B11, 5F3, 7H1, 6B5), produced by BIOS-Chile (Santiago, Chile), were selected.

Direct Anti-HBe and Anti-HBc Immunoassays

ELISA plates (Costar High Binding 96-well microtiter plates, Cambridge, MA) were coated separately with rHBeAg (Chiron) and rHBcAg (Chiron) at 2.0 μg/well in 1× phosphate buffered saline (PBS) overnight at 25°C. The rHBeAg was coated separately in its native state and its denatured state. To denature the antigen, 10 mM DTT and 0.2% SDS were added to the antigen. The mixture was then boiled for 5 min. To remove the DTT and reduce significantly the SDS concentration, the antigen was passed through a Sephadex G-50 Quick Spin column (Boehringer Mannheim, Indianapolis, IN) preequilibrated in 50 mM borate, pH 8.0, 0.2% gelatin, 0.05% Tween, and 0.09% sodium azide under centrifugation. The following day the plates were blocked with a post coat buffer (consisting of 1× PBS, 1% BSA, 0.02% sodium azide) for 1 hr at 25°C and then washed with deionized water. Sample diluent (190 μl) was added to designated wells of each plate, followed by 10 μl human sera or control human sera (positive and negative) and incubated for 1 hr at 37°C. The following day, plates were washed 5 times with wash buffer and 200 μl monoclonal mouse anti-human IgG:HRP was added to each well. Plates were incubated for 1 hr at 37°C, washed 5 times, and 200 μl of the substrate reagent consisting of OPD (o-phenylenediamine-2HCl, Sigma, St. Louis, MO) diluted in a citrate-phosphate buffer with 0.02% hydrogen peroxide was added to each well for color development. Plates were read at 490/620 nm dual wavelengths using an ELISA plate reader.

Neutralization Anti-HBe Immunoassays

The neutralization chemiluminescent immunoassays (CLIA) were carried out as follows. Fifty microliters of test sample was added to a 75 × 12 mm polystyrene tube (Sarstedt, Newton, NC), followed by 100 μl rHBeAg reagent, followed by 100 μl latex magnetic particles (LMPs; Bangs Laboratory, Inc., Fishers, IN) covalently linked to human anti-HBe antibodies. The tubes were then vortexed and incubated for 18 min in a 37°C water bath. Tubes were placed subsequently on a magnetic rack for 3 min to allow the LMPs to separate from solution. The unbound fractions were decanted and the aggregated LMPs were washed with 1 ml of wash buffer. This decant/wash cycle was repeated 2 additional times. At this point, 300 μl of the chemiluminescent tracer (human anti-HBe conjugated to dimethyacridinium ester (DMAE) was added to each tube. Again the tubes were vortexed, incubated for 18 min in a 37°C water bath, and placed on a magnetic rack for 3 min and washed as previously described. After the final decant, the tubes were loaded into an illuminometer (The Magic Lite Analyzer, Bayer Diagnostics, Walpole, MA). Each tube was then treated with a 300 μl aliquot of 0.5% hydrogen peroxide followed by a 300 μl aliquot of 0.25 N NaOH for light signal detection (measured in relative light units). The assay cutoff value was defined as 3 times the mean of 3 negative control sera for HBV infection. Abbott anti-HBe assays were run according to the manufacturer's specifications.

Peptide ELISA

ELISA plates (Costar High Binding 96-well microtiter plates) were coated with strepavidin (from *Streptomyces avidinii;* Boehringer Mannheim) at a concentration of 1.25 μg/well overnight at 25°C. The following day the plates were blocked with the ELISA post coat buffer for 1 hr each and then coated with biotinylated peptides at 2.0 μg/well overnight at 25°C. Sample diluent (190 μl) was added to each well of the peptide-coated plates, followed by serum samples (10 μl for human sera, 25 μl for mouse sera, or 25 μl for rabbit sera), and incubated for 20 hr at 25°C. Positive and negative controls were added for each type of sera (i.e., human, mouse, or rabbit). The following day plates were washed 5 times with wash buffer and 200 μl of the appropriate conjugate was added to each well (e.g., monoclonal mouse anti-human IgG:HRP for human sera, goat anti-mouse IgG:HRP for mouse sera, and goat anti-rabbit IgG:HRP for rabbit sera). Plates were then incubated for 1 hr at 37°C, washed 5 times, and 200 μl of the substrate reagent consisting of OPD diluted in a citrate-phosphate buffer with 0.02% hydrogen peroxide was added to each well for color development. Plates were read at 490/620 nm dual wavelengths using an ELISA reader.

TABLE I. Percent Reactivity of 114 HBsAg Positive Sera Samples to HBeAg Peptides

	HBV markers			Samples/	% Reactivity to peptides[a]			
Category	HBeAg	Anti-HBe	Anti-HBc	category	119–134	88–99	48–57	74–89
1	+	–	–	5	0.0	0.0	0.0	0.0
2	+	–	+	50	0.0	0.0	0.0	0.0
3	–	+	+	50	80.0	8.0	6.0	2.0
4	–	–	+	9	0.0	0.0	0.0	0.0

[a]% Reactivity = number of reactive samples/total number of samples in category × 100. Samples were considered reactive if the signal OD was 7 times or greater the average of 20 random HBV negative sera samples.

TABLE II. Reactivity of Anti-HBe and Anti-HBc mAbs With rHBeAg, rHBcAg, and HBeAg Peptides

	s/co[a]					
	rHBeAg Chiron	rHBcAg Chiron	Peptide 119–134	Peptide 88–99	Peptide 48–57	Peptide 74–89
Anti-rHBeAg mAbs						
8C71D3	2.6	0.3	1.9	0.3	0.4	0.6
8A5	2.3	0.6	2.4	0.5	0.5	0.5
8C72D2	3.8	0.0	3.4	0.0	0.4	0.0
7D10	3.3	0.0	1.3	0.1	0.1	0.5
5F3	2.4	0.5	0.3	4.7	0.5	0.5
2B11	2.7	2.2	3.0	0.4	0.7	0.4
7H1	1.0	0.7	0.3	0.4	0.8	0.6
6B5	1.7	2.1	0.6	0.3	0.9	0.8
Sorin e2	6.9	2.5	0.1	0.3	0.2	0.1
Anti-rHBcAg mAbs						
48F	2.0	1.8	1.4	0.3	0.4	0.4
3A3	0.1	4.1	0.2	0.2	0.2	0.2

[a]s/co = signal OD/cutoff OD. Cutoff OD = 3 × average of 3 HBV negative sera. A s/co of ≥1.0 is considered reactive.

RESULTS

Peptide Map

Three major linear epitope peaks spanning aas 49–56, 90–98, and 120–133 of the HBeAg sequence were revealed using the human anti-HBe-HRP conjugate included in the Abbott anti-HBe EIA kit (data not shown). Sequence 120–133 contains the HBeAg specific β epitope. Epitopes contained in sequences 90–98 and 49–56, however, have not been reported previously. These 2 novel epitopes were designated as δ and γ, respectively.

Mapping of Human and Rabbit Anti-HBe Sera

Human sera. One hundred fourteen HBsAg positive human sera samples, defined serologically by the Abbott Laboratories EIAs, were placed into 4 different categories according to their reactivities for 3 HBV markers: anti-HBe, anti-HBc, and HBeAg (Table I). These samples were tested on the 4 different peptides and gave the following results (Table I). The anti-HBe (–) sera from categories 1, 2, and 4 did not react to any of the peptides. In category 3 (the anti-HBe (+), HBeAg (–) sera), of the 50 samples tested, 40 (80%) reacted with peptide 119–134, 4 (8%) reacted with peptide 88–99, 3 (6%) reacted with peptide 48–57, and 1 (2%) reacted with peptide 74–89. As observed, only anti-HBe (+) sera reacted to the peptides, suggesting that the epitopes contained in these peptide sequences are HBeAg specific. A positive correlation was also observed between anti-HBe antibody titer and antibody titer to peptide 119–134 (data not shown). This correlation was observed previously [Sallberg et al., 1991]. The results confirm that the major linear epitope [Sallberg et al., 1991] resides in the aa region 119–134.

Rabbit anti-HBe. Of the 4 different rabbit antisera to rHBeAg, 2 reacted with peptide 119–134, and all 4 reacted to peptide 74–89 (data not shown). Peptides 88–99 and 48–57 were not recognized by the rabbit antisera. These results imply that the rabbit immune response is directed against both regions 119–134 and 74–89, but does not recognize regions 88–99 and 48–57.

Specificity and Mapping of mAbs

Six of the mAbs derived from rHBeAg (8C71D3, 8A5, 8C72D2, 7D10, 5F3, 7H1) reacted exclusively with rHBeAg, whereas 2 (2B11, 6B5) reacted with both rHBeAg and rHBcAg (Table II). The Sorin anti-HBe detection mAb (e2) was also found to react with both rHBcAg and rHBeAg. Anti-HBe mAbs 8C71D3, 8A5, 8C72D2, 7D10, and 2B11 reacted exclusively with peptide 119–134 (containing the β epitope), whereas 5F3 reacted exclusively with the peptide 88–99 (containing the δ epitope) (Table II). The remaining 2 mAbs (7H1, 6B5) did not react to any of the peptides. These antibodies and the Sorin e2 antibody (a known conformational anti-HBe mAb to the HBe2 epitope) were then

TABLE III. Reactivity of mAbs With Native and Denatured HBV Recombinant Antigens

	OD				% Loss of reactivity[a]	% Loss of reactivity[b]
	rHBeAg	drHBeAg	rHBcAg	drHBcAg		
Anti-rHBeAg						
8C72D2	1.97	1.95			0.0	
6B5	2.58	1.09			58.8	
7H1	1.33	0.37			75.6	
Sorin e2:HRP	1.73	0.35			79.1	
Anti-rHBcAg						
48F			1.649	1.219		26.1
3A3			1.270	0.154		87.9

[a]% Loss of reactivity = (OD to rHBeAg – OD to drHBeAg)/OD rHBeAg × 100.
[b]% Loss of reactivity = (OD to rHBcAg – OD to drHBcAg)/OD rHBcAg × 100.

tested for their ability to bind to denatured rHBeAg in order to determine if these 2 antibodies were conformational (Table III). Antibodies 7H1, 6B5, and e2 showed a 76%, 59%, and 79% loss of reactivity, respectively, to denatured rHBeAg compared with native rHBeAg, indicating that antibodies 7H1 and 6B5 were also conformational (Table III). The mAb was considered conformational if a 50% or greater loss of reactivity was observed. As a control, anti-HBe mAb 8C72D2, which was mapped to the linear region 119–134, was also tested. There was no difference in reactivity observed in its ability to bind native or denatured rHBeAg.

Of the 2 mAbs derived from rHBcAg, 3A3 reacted exclusively with rHBcAg whereas 48F reacted with both rHBcAg and rHBeAg (Table II). Antibody 48F reacted exclusively with peptide 119–134. Antibody 3A3 did not react to any of the peptides. This antibody was then tested for its ability to bind denatured rHBcAg and showed an 88% loss of reactivity to denatured rHBcAg compared with native rHBcAg, indicating that this mAb was conformational. Antibody 48F was tested as a control on both native and denatured rHBcAg and displayed only a 26% loss of reactivity to denatured rHBcAg compared with native rHBcAg (Table III).

Taken together, these mAbs confirm the existence of the β epitope, the novel identified δ epitope, an HBeAg conformational epitope, and an HBcAg specific conformational epitope.

Ability of Anti-HBe mAbs to Capture Native HBeAg

Seven of the anti-HBe mAbs, which were all efficient at detecting rHBeAg, were tested for their ability to either capture or detect HBeAg when coupled to LMPs or conjugated to DMAE and used in a sandwich CLIA (data not shown). Two of these mAbs (8C72D2, 7D10), both mapped to region 119–134, were conjugated to DMAE. These mAbs were able to detect only 20–50% of the serum HBeAg detected by polyclonal human anti-HBe when tested against a set of HBeAg positive samples (data not shown). mAbs 5F3, 6B5, and 7H1 were coupled to LMPs and used in a HBeAg capture assay. mAb 5F3, mapped to aa region 88–99, was only able to capture approximately 10% of HBeAg in samples captured by the polyclonal human anti-HBe (data not shown). However, mAbs 7H1 and 6B5, which were found to be conformational, were able to capture 96% and 100%, respectively, of the HBeAg present in a set of HBeAg positive samples compared with the human anti-HBe (data not shown). These results suggest that conformational mAbs to rHBeAg are more efficient at capturing serum HBeAg than are linear anti-HBe mAbs.

Anti-HBc and Anti-HBe Immunoassays

Anti-HBe seroconversion panels (SCPs). HBV anti-HBe SCPs A, B, and C were tested by 11 different assays (Table IV). The assays used were an anti-HBc ELISA using rHBcAg (rHBcAg ELISA), Abbott anti-HBc EIA, anti-HBe ELISA using rHBeAg (rHBeAg ELISA), anti-HBe ELISA using denatured rHBeAg (drHBeAg ELISA), our anti-HBe neutralization CLIA (Chiron CLIA), Abbott anti-HBe neutralization EIA (Abbott EIA), the 4 different HBeAg peptide ELISAs, and the Abbott HBeAg (Table IV). In SCPs A and B, anti-HBc was detected by the rHBcAg ELISA earlier than anti-HBe was detected by the rHBeAg ELISA, emulating the serologic profile for anti-HBc and anti-HBe in HBV infection. In all 3 SCPs, the rHBeAg ELISA had significantly greater sensitivity than both the Abbott EIA and the Chiron CLIA. The rHBeAg ELISA was able to detect anti-HBe up to 4 bleed dates earlier than the Abbott EIA and the Chiron CLIA. Anti-HBe antibodies in bleeds 10 and 11 of SCP A were detected by the rHBeAg ELISA, but not by the Abbott anti-HBe EIA. To determine the specificity of these earlier detected antibodies, these samples were tested against the HBeAg peptides. The anti-HBe antibodies in these 2 samples reacted with the anti-HBe specific peptide 119–134, demonstrating the presence of anti-HBe specific antibodies. Anti-HBe antibodies in sample 11 were also detected by the Chiron CLIA. This demonstrates that the rHBeAg ELISA therefore has the capacity to detect anti-HBe specific antibodies in samples that contain moderate to excess HBeAg, whereas the Chiron CLIA is able to detect anti-HBe in samples that contain moderate HBeAg levels. Anti-HBe antibodies present in bleeds 7–8 of SCP A, bleed 16 of SCP B, and bleeds 8–11 of SCP C were all detected by the rHBeAg ELISA, but not by the Abbott anti-HBe EIA. These samples did not react with either

TABLE IV. Anti-HBe Seroconversion Panels (SCP) Tested on HBV Antigens and HBeAg Peptides*

	Anti-HBc assays		Anti-HBe assays				Peptide ELISA: HBeAg aa sequence				HBeAg assay
	rHBcAg ELISA s/co[a]	Abbott EIA co/s	rHBeAg ELISA s/co[a]	drHBeAg ELISA s/co[a]	Chiron CLIA co/s[b]	Abbott EIA co/s	119–134 s/co[a]	88–99 s/co[a]	48–57 s/co[a]	74–89 s/co[a]	Abbott EIA s/co
SCP A											
5	**14.0**	0.9	0.1	0.1	0.4	0.3	0.1	0.2	0.1	0.2	21.5
6	14.4	**1.1**	0.1	0.0	0.3	0.3	0.1	0.2	0.1	0.2	19.8
7	17.9	3.3	**1.3**	0.0	0.6	0.3	0.1	0.2	0.1	0.1	21.2
8	26.6	6.7	1.9	0.0	0.7	0.3	0.1	0.2	0.1	0.2	18.9
9	22.9	25.0	4.6	0.3	0.6	0.3	0.9	0.4	0.3	0.3	25.7
10	26.8	11.1	10.0	**1.8**	0.5	0.3	**1.3**	0.4	0.2	0.3	19.6
11	23.7	12.5	8.2	2.2	**1.0**	0.6	1.4	0.3	0.2	0.2	6.3
12	25.1	14.3	7.9	3.6	1.7	**1.3**	1.2	0.3	0.2	0.2	1.0
SCP B											
13	0.3	**2.2**	0.1	0.2	0.4	0.3	0.1	0.2	0.2	0.1	18.7
14	**1.4**	5.8	0.1	0.1	0.3	0.3	0.0	0.3	0.1	0.1	20.8
15	7.5	9.4	0.5	0.2	0.3	0.3	0.1	0.1	0.1	0.1	19.0
16	27.3	14.0	**2.8**	0.5	0.5	0.3	0.2	0.2	0.3	0.1	19.0
17	23.8	22.8	8.9	**1.5**	**1.4**	**1.2**	0.5	0.4	0.2	0.4	0.4
18	25.2	16.6	8.9.	2.1	1.0	1.3	0.6	0.4	0.3	0.2	0.3
19	25.0	22.8	8.1	3.0	1.3	1.4	**1.1**	0.4	0.2	0.3	0.3
SCP C											
8	**1.3**	**1.3**	**3.1**	0.1	0.8	0.7	0.1	0.0	0.0	0.0	1.9
9	5.1	2.2	6.6	0.8	**1.1**	**1.4**	0.7	0.0	0.0	0.0	0.3
10	5.1	5.0	8.4	0.6	1.3	1.8	0.7	0.0	0.0	0.0	0.3
11	5.5	5.3	7.1	0.5	1.2	1.6	0.4	0.0	0.0	0.0	0.3
12	7.0	5.3	9.9	**1.6**	2.1	1.7	0.6	0.1	0.0	0.0	0.2
13	7.0	6.3	11.4	2.3	4.1	2.9	**1.6**	0.0	0.0	0.0	0.2

*s/co and co/s for all Abbott EIAs above were determined according to the manufacturer's specifications. A s/co or a co/s of ≥1.0 is considered reactive.
[a]s/co for ELISA = signal OD/cutoff OD. Cutoff OD = 4 × average of 3 HBV negative sera.
[b]co/s for Chiron anti-HBe CLIA = cutoff OD/signal OD. Cutoff OD = (average of 2 HBV anti-HBe positive controls + average of 3 HBV negative controls)/2.

the drHBeAg ELISA or the HBeAg peptides. This would suggest that these anti-HBe antibodies detected early are conformational.

Detection of anti-HBe in selected HBsAg (+) samples. Samples 1 and 2 (Table V), which were both HBeAg (+), anti-HBc (+), and anti-HBe (–) as measured by the Abbott EIAs, were anti-HBe positive by the rHBeAg ELISA and on our neutralization anti-HBe assay. These samples were also reactive by the drHBeAg ELISA. Sample 2 displayed reactivity with peptide 119–134, indicating the presence of anti-HBe specific antibodies in this sample. Sample 1, however did not react with any of the peptides, suggesting that the anti-HBe present in this sample may recognize an undefined HBeAg linear epitope. In order to test whether the Abbott rHBeAg was also capable of being neutralized by these samples when present at higher concentrations, the Abbott Neutralization Reagent (containing rHBeAg) was concentrated 3-fold (3×). With the Abbott rHBeAg at 3×, samples 1 and 2 showed borderline to complete neutralization (Table V).

DISCUSSION

Mapping Epitopes With Selected Peptides

Of the anti-HBe (+) samples tested, 80% reacted with peptide 119–134, indicating an HBeAg specific epitope contained in this sequence. The fact that peptides 88–99 and 48–57 were reactive with anti-HBe (+) samples, but nonreactive with anti-HBe (–) samples, indicates that these 2 epitopes are also HBeAg specific. Although it has been reported that peptide 74–84 is reactive with anti-HBc (+), anti-HBe (–) sera [Tordjeman et al., 1993], in our study peptide 74–89 only reacted with anti-HBc (+), anti-HBe (+) sera but not with anti-HBc (+), anti-HBe (–) sera. The reason for this is unknown, however, it was also reported that peptide 74–84 is only poorly reactive to anti-HBc (+) sera [Tordjeman et al., 1993]. Although all 3 SCPs showed reactivity to peptide 119–134, these panels did not show reactivity to the other 3 linear epitopes. Reactivity to the other 3 peptides was only found in samples that were HBeAg (–) and typically contained high anti-HBe titers. This would indicate that antibodies to these linear epitopes are more typically found in samples that carry a high anti-HBe titer and are negative for HBeAg.

mAbs

Both human and murine humoral immune systems recognized the linear region 119–134 as a dominant linear epitope. It was surprising that mAb 2B11, derived from rHBeAg, and mAb 48F, derived from rHBcAg, were crossreactive with both antigens and were found to recognize the HBeAg specific region 119–134. A possible explanation for this is that our rHBcAg lacks the structural integrity of native HBcAg and may exist in particle and dimeric and/or monomeric forms. This rHBcAg has been found to form a nucleocapsid particle by wrapping around the RNA present in

TABLE V. Detection of Anti-HBe in Selected HBsAg (+) Samples*

	Anti-HBc	Anti-HBe assays					Peptide ELISA: HBeAg aa sequence				HBeAg
	Abbott EIA co/s	rHBeAg ELISA s/co[a]	drHBeAg ELISA s/co[a]	Chiron CLIA co/s[b]	Abbott EIA 1× NR co/s	Abbott EIA 3× NR co/s	119–134 s/co[a]	88–99 s/co[a]	48–57 s/co[a]	74–89 s/co[a]	Abbott EIA s/co
Sample 1	30.9	7.8	3.5	3.1	0.4	**0.9**	0.5	0.4	0.4	0.1	11.5
Sample 2	16.7	6.9	11.1	1.4	0.7	**1.2**	1.3	0.8	0.4	0.4	3.5

*s/co and co/s for all Abbott EIAs above were determined according to the manufacturer's specifications. A s/co or a co/s of ≥1.0 is considered reactive. NR = neutralization reagent (rHBeAg) in the Abbott anti-HBe EIA kit.
[a]s/co for ELISA = signal OD/cutoff OD. Cutoff OD = 4 × average of 3 HBV negative sera.
[b]co/s for Chiron anti-HBe CLIA = cutoff OD/signal OD. Cutoff OD = (average of 2 HBV anti-HBe positive controls + average of 3 HBV negative controls)/2.

the yeast cell cytoplasm. Because native HBcAg forms the nucleocapsid particle by wrapping around HBV viral DNA it may be more intact. In the dimeric form, the β epitope could be exposed, allowing its recognition by mAbs to this epitope. It was also observed that both the Sorin anti-HBe detection mAb, mapped to the conformation HBe2 epitope [Salfeld et al., 1989], and our conformational anti-HBe mAb (6B5) recognized both rHBeAg and rHBcAg. It was reported previously that the Sorin anti-HBe detection mAb recognized rHBeAg but did not recognize native HBcAg [Salfeld et al., 1989]. If our rHBcAg exists in both particulate and dimeric forms, this could explain why these conformational anti-HBe mAbs also recognize our rHBcAg.

Comparison of rHBeAg With Human Serum HBeAg

Two pieces of data would indicate that rHBeAg is not antigenically identical to serum HBeAg. First, anti-HBe present in human samples that contained excess HBeAg was able to neutralize both our rHBeAg and the Abbott rHBeAg when the rHBeAg is concentrated in the Abbot anti-HBe EIA, but these anti-HBe antibodies were unable to neutralize the serum HBeAg present in these samples (Table V). Second, anti-HBe mAbs mapped to the β and δ epitopes were very efficient in detecting rHBeAg but were inefficient in detecting serum HBeAg. These data suggest that the β and δ epitopes present on serum HBeAg are not as accessible as they are on rHBeAg. Because serum HBeAg has been found to exist in different forms (i.e., a large MW form bound to IgG and a small MW, IgG-free polypeptide) and as a heterogeneous population of different lengths, these 2 epitopes may not be accessible in all forms and lengths of serum HBeAg.

The conformational epitope recognized by the Sorin e2 mAb and the conformational epitope(s) recognized by our anti-HBe mAbs (6B5, 7H1), however, seem to be accessible in all forms of serum HBeAg because these mAbs were able to detect both rHBeAg and serum HBeAg as efficiently as human anti-HBe.

Immunoassay Format

The Abbott and Sorin anti-HBe EIAs employ an assay format such that anti-HBe is only detectable in the absence of excess HBeAg. The reason for this limitation lies in the design of the assay. Both of these commercial assays employ a rHBeAg, which can be neutralized in the presence of anti-HBe. The rHBeAg employed in these assays is present at a very low concentration (approximately 25 ng/ml). This concentration may have been used in order to enable the assay to detect very low anti-HBe titers capable of neutralizing the rHBeAg. Neutralization of the rHBeAg by anti-HBe prevents its binding to the solid phase coupled to anti-HBe and the detecting antibody (anti-HBe:HRP). Therefore, a negative result indicates a sample positive for anti-HBe. If, however, a sample contains anti-HBe but also contains a significant level of serum HBeAg that is not completely neutralized, the rHBeAg could be neutralized but would be masked by the level of serum HBeAg present in the sample, which continues to maintain the OD. In other words, this type of sample would give a significant OD value, thereby appearing negative. We have identified such samples (Table V). These samples contain moderate to excess HBeAg levels and are anti-HBc (+). When tested on the Abbott anti-HBe EIA they appear to be anti-HBe (–). If, however, the rHBeAg in the Abbot EIA is concentrated, giving a higher rHBeAg concentration than the serum HBeAg concentration, then these samples display neutralization. This phenomenon was also observed using the Sorin anti-HBe EIA when testing against 1 of these 2 samples (data not shown).

Another reason Abbott may have designed their assay with this limitation may have a clinical implication. A patient who is HBeAg positive is considered highly infectious. If an anti-HBe assay is able to detect anti-HBe in the presence of excess HBeAg, this result could cause the clinician to assume that, because the patient has seroconverted, he or she will necessarily clear the HBeAg in a relatively short time period. Our results show that anti-HBe is found often in patients seropositive for HBeAg. It also has been reported previously that anti-HBe can exist for many years in HBV chronic patients seropositive for HBeAg [Maruyama et al., 1993]. On the other hand, the capacity to detect anti-HBe in the presence of excess HBeAg could have a clinical significance as an early indicator of response to a particular immunotherapy (e.g., αIFN) for HBV chronically infected individuals [Evans et al., 1997].

A possible reason for the neutralization assay design

as opposed to a direct anti-HBe assay could be to ensure that the anti-HBe detected is truly anti-HBe specific and that the assay is not merely detecting HBcAg/HBeAg cross-reactive antibodies. Neutralization of the rHBeAg must occur for a positive anti-HBe result, and neutralization can only occur if a sample contains a sufficient heterogeneity of anti-HBe capable of saturating the different epitopes necessary to neutralize the rHBeAg. If a sample contains only anti-HBe antibodies that recognize shared HBeAg/HBcAg epitopes it would potentially be detectable by a direct format but these antibodies may not be sufficient for neutralization. A neutralization assay design is therefore more likely than a direct anti-HBe design to ensure that anti-HBe specific antibodies are present in a sample that is reactive in the assay.

Because the neutralization assay format seems to be scientifically more sound for the detection of anti-HBe specific antibodies, the best solution to detecting anti-HBe specific antibodies in the presence of excess HBeAg could be to increase the rHBeAg concentration in these assays, thus allowing earlier detection of anti-HBe.

Chiron rHBeAg vs. Abbott and Sorin rHBeAgs. Because the Abbott and Sorin anti-HBe EIAs were used as a reference for our anti-HBe assays in this study, it was important to ensure that the rHBeAg and both the Sorin and Abbott rHBeAgs were antigenically identical. Our rHBeAg was substituted at 25 ng/ml in both Sorin and Abbot anti-HBe EIAs and tested against a well-pedigreed anti-HBe mixed titer panel. Our results matched the Abbott and Sorin anti-HBe results for this panel (data not shown).

Anti-HBc and anti-HBe serologic profiles. The anti-HBc and anti-HBe results for the SCPs given by the ELISA assays (Table IV) show anti-HBc detection earlier than anti-HBe. It could be argued that this is merely due to differences in assay sensitivity rather than a demonstration of earlier anti-HBc appearance than anti-HBe in the serologic profile. Although this is possible, it is unlikely. Both rHBeAg and rHBcAg were coated at 2 μg/well. The MW of our rHBcAg polypeptide is 22 kDa. It exists primarily as a particle of 240 dimer subunits with a combined particle MW of 10,560 kDa. Our rHBeAg-SOD fusion has a MW of 34.5 kDa and exists as a monomer. Therefore, there were approximately 306-fold more individual rHBeAg molecules than rHBcAg particulate molecules coated on the ELISA plates. Molecule per molecule, this would allow the rHBeAg epitopes to be more accessible than the rHBcAg epitopes. Therefore, when comparing sensitivity one would predict the opposite, that sensitivity should favor the rHBeAg-coated plate.

ACKNOWLEDGMENTS

We gratefully acknowledge Darrell Peterson for supplying us with rHBeAg, and Steve Tirrell, Wanda Zeigler, and Nick Masiello for providing us with PMP and DMAE research reagents.

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